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Inhibition of Tumor-induced Angiogenesis by Ribonuclease Inhibitor

Ken-ichi NORIOKA, Toshihiro MITAKA and Yohichi MOCHIZUKI

*Cancer Research Institute, Department of Pathology, Sapporo Medical
College, South 1, West 17, Chuo-ku, Sapporo 060, JAPAN*

ABSTRACT

The effect of recombinant ribonuclease inhibitor on angiogenesis induced by B16-F10 cells was examined in syngeneic mice. Ribonuclease inhibitor coated with a slow release substance caused a marked decrease of the number of vessels toward tumor mass (angiogenic response), however, injection of ribonuclease inhibitor did not. Ribonuclease inhibitor did not directly affect the growth of tumor cell or endothelial cell *in vitro*. These results suggest that ribonuclease inhibitor could be an inhibitor of tumor-induced angiogenesis by using an appropriate delivery system and stabilizing its activity.

Key words: Angiogenesis, Ribonuclease inhibitor, Angiogenin,
Vascular endothelial cells.

INTRODUCTION

Angiogenesis, the biological process of forming new blood vessels, is highly regulated under normal conditions. However, in numerous pathological states, this regulation fails as a result the disease itself is modified by persistent, unabated neovascularization. These diseases include rheumatoid arthritis, diabetic retinopathy, psoriasis and tumor development (1,2). Especially, solid tumor growth and metastasis are believed to be angiogenesis-dependent (3), and in the absence of angiogenesis, these phenomena are circumscribed, leading to tumor dormancy. Consequently, the regulation of angiogenesis could be a novel strategy for therapeutical use. This concept of "antiangiogenic therapy" stimulated the search for angiogenesis inhibitors, and within the past few years, many inhibitors have been identified, including protamine (4), corticosteroid (5), inter-

The abbreviations used are : PRI, placental ribonuclease inhibitor; MAE, mouse aortic endothelial cells; FBS, fetal bovine serum; DMEM, Dulbecco's modification of Eagle's Minimum Essential Medium; EVA, ethylene vinyl acetate; FGF, fibroblast growth factor.

feron (6), platelet factor IV (7), thrombospondin (8), sulfated chitin derivatives (9), antibiotics (10) and metalloproteinase inhibitor (11). These agents have been directed against various aspects of the neovascular process: blocking endothelial cell migration and/or proliferation, inhibiting the proteolytic degradation of extracellular matrix components of endothelial cells.

Pancreatic ribonuclease inhibitors have long been known to be present in mammalian tissues, and one of these, isolated from human placenta (placental ribonuclease inhibitor; PRI), has been studied most extensively (12). PRI binds to ribonuclease, and recently it is reported to bind to angiogenin, which is one of the most potent angiogenesis-inducer and its structure is highly homologous to that of the pancreatic ribonuclease (13,14). Indeed, PRI abolishes both angiogenic and ribonucleolytic activities of angiogenin using chick embryo chorioallantoic membrane assay (15). In this study, we examined the potential efficacy of ribonuclease inhibitor as an anti-cancer drug, using a cell proliferation assay *in vitro* and a tumor-induced angiogenesis assay in a mouse model.

MATERIALS AND METHODS

Reagents

Lyophilized recombinant ribonuclease inhibitor (100 U/ μ g, RNasin^T) was supplied by Promega Corporation (Madison, USA). This product is equivalent to native PRI in structure and in its ability to inactivate ribonuclease. Endotoxin level was under 0.06 eu/ml measured by the Limulus test.

Cell culture

Mouse aortic endothelial cells (MAE) were obtained from BALB/c mice by collagenase penetration as described previously (16). Endothelial cells were purified by cell sorting using a monoclonal antibody to angiotensin converting enzyme. MAE were grown in Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, USA).

The B16-F10 melanoma subline was kindly provided by Dr. I.J. FIDLER (Anderson Cancer Center, Houston, USA). Melanoma cells were cultured in DMEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, non-essential amino acids, and L-glutamine.

Assay for cell growth

Cells were seeded at 1×10^4 in 24 multiwell plates (Becton Dickinson, Oxnard, USA). After overnight incubation, test reagents were added to the cultures. At subsequent time points, cells were harvested by trypsinization and were count-

ed by using an electronic particle counter (Coulter Electronics, Hialeah, USA).

Assay for tumor-induced angiogenesis

The assay for tumor-induced angiogenesis in syngeneic mice was carried out as previously described (17). C57BL/6 mice were inoculated intradermally with B16-F10 melanoma cells (5×10^5) on the right and left side at the same level of the body axis. After 3 days, PRI was administered by intratumoral injection or by implantation of slow-release formulations. In brief, PRI was absorbed in polyvinyl alcohol sponge (Kanebo, Japan), and the coated with ethylene vinyl acetate (EVA) copolymer (DuPont, USA), was implanted subcutaneously under the site. After 3 days, the number of vessels oriented toward the tumor mass were determined by a single observer in a blinded manner. The tumor size was approximated by averaging the diameters of short and long axis of the mass.

Statistical analysis

The statistical significance of differences between groups was calculated by applying the two tailed paired *t* test.

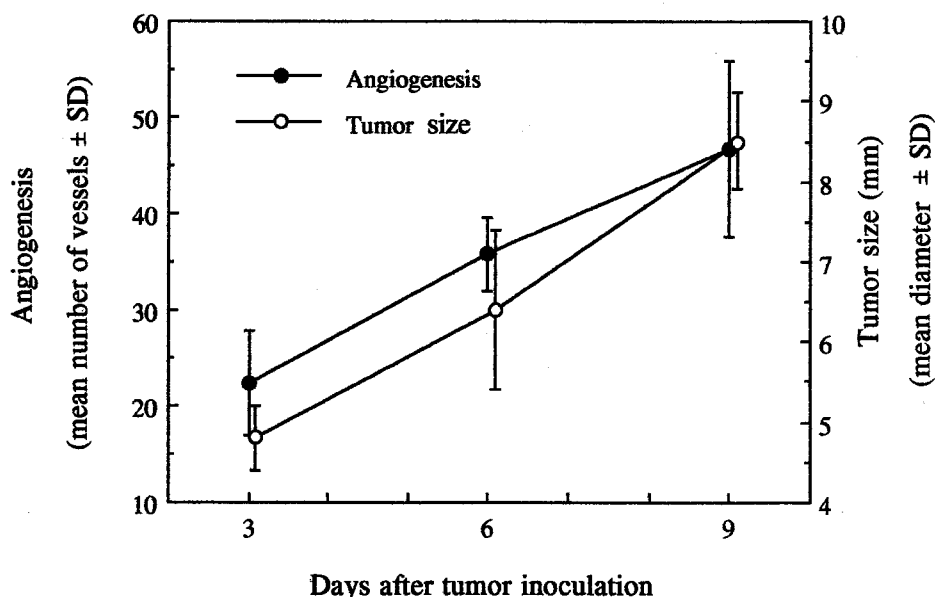


Fig. 1 Time course of angiogenesis and tumor growth at tumor injection sites. Three C57BL/6 mice per group were inoculated intradermally with B16-F10 cells (5×10^5) at two sites on the back. At various days after tumor inoculation, mice were killed and the skin was separated from underlying tissues. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass.

RESULTS

Angiogenesis and tumor growth

Tumor-induced angiogenesis contributes to the growth of solid tumors (3). We first examined this phenomenon by injection of B16-F10 melanoma cells on the back of syngeneic mice. Fig. 1 shows that angiogenic response (number of vessels) at the tumor injection site increased in a time dependent manner and was proportional to the tumor growth (tumor size) within 9 days after tumor inoculation. Thereafter, vessels became dilatated, but little change in the number of vessels, was observed. The background vascularity was observed around 6-13 vessels at the site before injection.

Intratumor injection of PRI

Next we examined the effect of PRI in this melanoma system to determine the ability of PRI to inhibit tumor growth and tumor-induced angiogenesis. Since initial experiments using intravenous administration of PRI showed no effect in this tumor model, local administration of PRI was carried out. However, even high doses of PRI injected intratumorally did not inhibit the size of the tumor (Fig. 2A) nor the number of induced vessels (Fig. 2B).

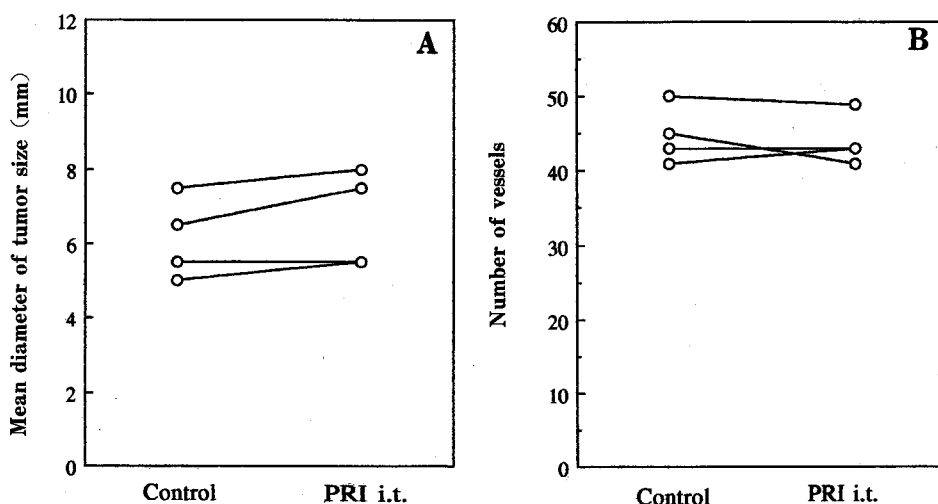


Fig. 2 Effect of PRI injected i.t. on tumor growth and angiogenesis. B16-F10 melanoma cells were injected intradermally into ventral skin of C57BL/6 mice. After 3 days, 10000 U of PRI or PBS (control) were injected at the sites. After 3 days, mice were killed and the injection sites were examined via peritoneal surface for tumor implant size (A) and centrally directed tumor angiogenesis (B).

Administration of PRI within a slow-release pellet

Since ribonuclease inhibitor is labile and unstable in the serum, we introduced PRI into the central EVA copolymer-coated core of a polyvinyl sponge and tested it as a subcutaneous implant. Although the tumor growth (size) was not affected by this treatment (Fig. 3A), neovascularization was significantly decreased in comparison with control ($p < 0.01$, Fig. 3B).

Effect of PRI on cell growth in vitro

Endothelial cell growth is an important step in neovascularization. We tested the influence of PRI on the growth of endothelial cells *in vitro*. Fig. 4 shows that even at 10000 U/ml PRI did not affect MAE cell growth after a 48-h incubation period. Similarly, the incubation of B16-F10 cells with PRI did not affect their growth (Fig. 5).

DISCUSSION

We have demonstrated here that PRI in a slow release substance inhibited tumor-induced angiogenesis in syngeneic mice. However, PRI did not affect the growth of tumor cells and MAE cells *in vitro*. These results suggest that PRI

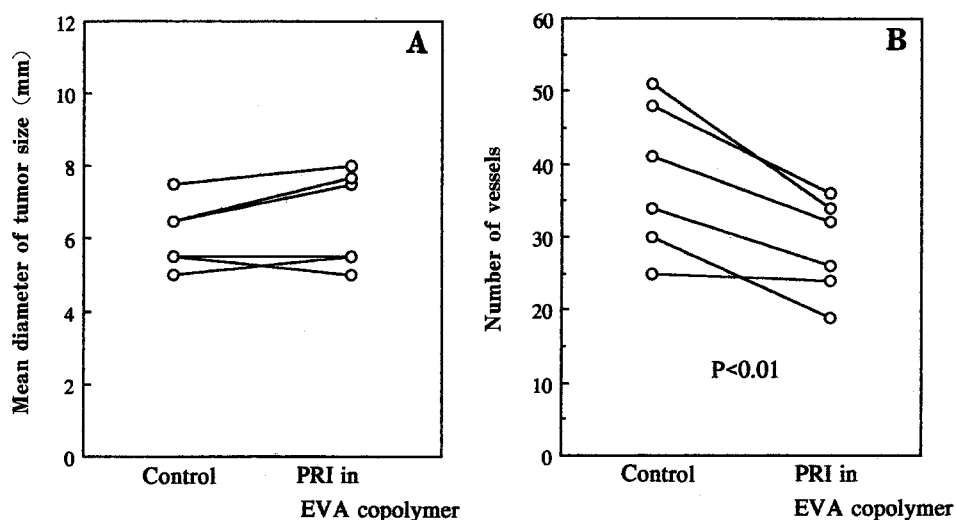


Fig. 3 Effect of PRI in EVA copolymer on tumor growth and angiogenesis. B16-F10 melanoma cells were injected intradermally into ventral skin of C57BL/6 mice. After 3 days, 10000 U of PRI or PBS (control) dispersed within a EVA copolymer were implanted underneath intradermally the sites. After 3 days, mice were killed and injection sites were examined via peritoneal surface for tumor implant size (A) and centrally directed tumor angiogenesis (B).

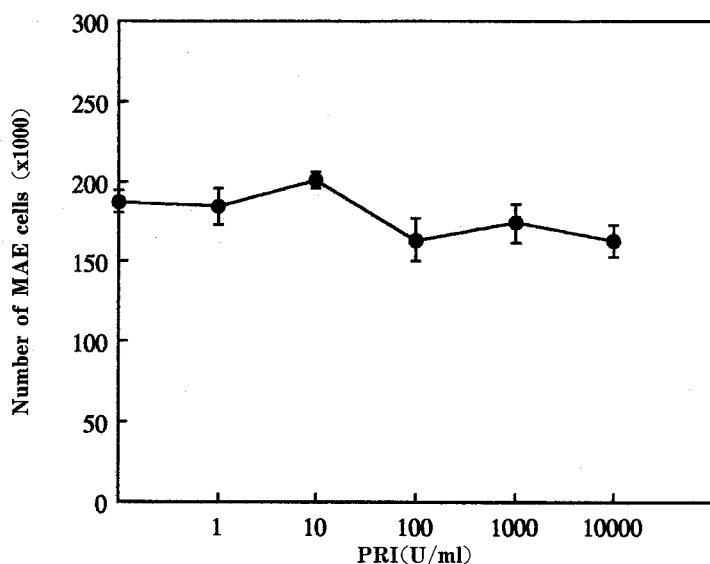


Fig. 4 Effect of PRI on growth of MAE cells. Cells were cultured with the medium containing 5% of fetal calf serum and were seeded in 24 wells. PRI concentrations indicated was added every day. After 3 days-incubation, cells were harvested by trypsinization and were counted. Results are expressed as mean \pm SD of quadruplicates cultures.

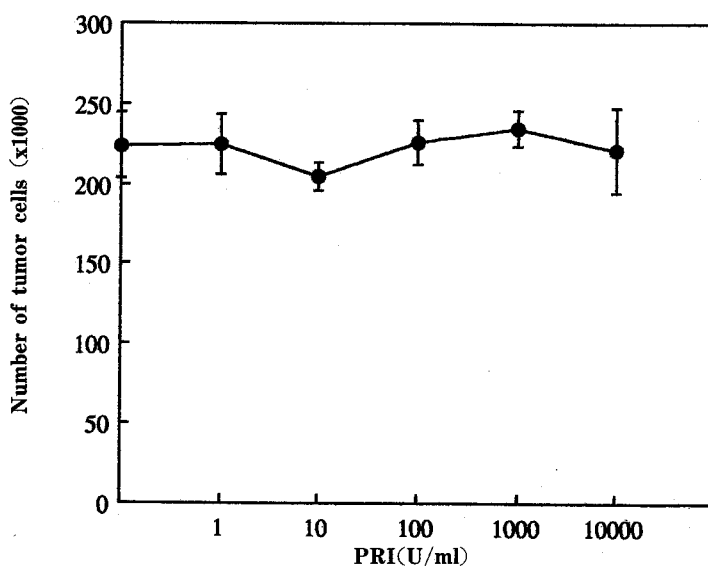


Fig. 5 Effect of PRI on growth of B16-F10 melanoma cells. Cells were cultured with the medium containing 5% of fetal calf serum and were seeded in 24 wells. PRI concentrations indicated was added every day. After 2 day-incubation, cells were harvested by trypsinization and were counted. Results are expressed as mean \pm SD of quadruplicates cultures.

may prevent the formation of new capillary vessels induced by tumor without affecting the growth of vascular endothelial cells and tumor cells directly.

Shapiro and Vallee have shown that PRI abrogates the ability of human tumor cell line derived-angiogenin to induce neovascularization in the chick embryo chorioallantoic membrane assay (15). Angiogenin is not a mitogen for vascular endothelial cells, but it is one of the most potent angiogenesis-inducing molecules (13). Recently, angiogenin has been reported to support endothelial cell adhesion, which may be a critical step in the process of angiogenesis (18). Although it is not yet clear whether angiogenin has a central role for tumor-induced angiogenesis, the simplest assumption is that inhibition of the tumor-induced angiogenesis by PRI is associated with blocking the activity of angiogenin.

Fibroblast growth factor (FGF) is a different type of angiogenesis inducer. In contrast with angiogenin, FGF is a potent mitogen for vascular endothelial cells, but its structure lacks a signal sequence (19). Although invalidity of the antibodies against FGF for tumor angiogenesis has been reported (20), it is reported that specific antibody against FGF suppressed solid tumor growth *in vivo*, in which tumor cells had been transfected with the leader sequence-fused basic FGF gene (21). The ordinary mechanism of releasing FGF from cells is yet unknown, but it has been suggested that angiogenin might induce vascularization by effecting the release of FGF from cells (22). Interestingly, preliminary experiments by Polakowski *et al.* showed that addition of PRI to FGF-containing cores reduced the angiogenic reaction in the disc angiogenesis system in mice (23). The relation between FGF and PRI remains to be investigated. Other possibilities also remain that PRI exerts its effect on angiogenesis by several distinct pathways, because angiogenesis is a multi-step process.

In summary, our experiments indicate that PRI could be an inhibitor of tumor-induced angiogenesis by coating with a slow release substance. Although PRI did not suppress the tumor growth in this study, the anti-angiogenic activity of PRI might be of benefit for preventing the early phase of tumor development including metastasis, and other angiogenic diseases.

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